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Transport of β-Casein-derived Peptides by the Oligopeptide Transport System Is a Crucial Step in the Proteolytic Pathway of *Lactococcus lactis**

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In the proteolytic pathway of *Lactococcus lactis*, milk proteins (caseins) are hydrolyzed extracellularly to oligopeptides by the proteinase (PrtP). The fate of these peptides, *i.e.* extracellular hydrolysis followed by amino acid uptake or transport followed by intracellular hydrolysis, has been addressed. Mutants have been constructed that lack a functional di-tripeptide transport system (DtpT) and/or oligopeptide transport system (Opp) but do express the P_1-type proteinase (specific for hydrolysis of β- and to a lesser extent κ-casein). The wild type strain and the DtpT$^-$ mutant accumulate all β-casein-derived amino acids in the presence of β-casein as protein substrate and glucose as a source of metabolic energy. The amino acids are not accumulated significantly inside the cells by the Opp$^-$ and DtpT$^-$ Opp$^-$ mutants. When cells are incubated with a mixture of amino acids mimicking the composition of β-casein, the amino acids are taken up to the same extent in all four strains. Analysis of the extracellular peptide fraction, formed by the action of PrtP on β-casein, indicates that distinct peptides disappear only when the cells express an active Opp system. These and other experiments indicate that (i) oligopeptide transport is essential for the accumulation of all β-casein-derived amino acids, (ii) the activity of the Opp system is sufficiently high to support high growth rates on β-casein provided leucine and histidine are present as free amino acids, and (iii) extracellular peptidase activity is not present in *L. lactis*.

Lactic acid bacteria possess an active proteolytic system that is involved in the degradation of milk proteins (α_{S1}-, α_{S2}-, κ-, and β-casein). Most of the amino acids released from the hydrolysis of caseins are essential or growth stimulating. The proteolytic system of *Lactococcus lactis* consists of a proteinase, several peptidases, amino acid transporters, and two peptide transport systems. The extracellularly located proteinase (PrtP) performs the first step in the degradation of caseins and is essential for growth on milk to high cell densities. The total peptide formation resulting from the action of the P_1-type proteinase on β-casein has been analyzed *in vitro* using purified proteinase.[1] This study has indicated that β-casein is degraded to fragments of 4–30 residues, of which 17% is smaller than 9 residues. None of the peptidases studied to date possesses an amino-terminal signal that could target the protein to the outside of the cell (Pritchard and Coolbear, 1993; Kok and De Vos, 1993). Furthermore, biochemical and immunological data indicate that the aminopeptidases (PepN and PepC), the *X*-prolyl-dipeptidyl aminopeptidase (PepX), the endopeptidase (PepO), the tripeptidase (PepT), and the glutamyl aminopeptidase (PepA) are present inside the cell (Tan *et al.*, 1992; Baankreis, 1992). In view of these observations, it would seem that the casein-derived peptides have to be taken up by the cells before further hydrolysis can take place. Two peptide transport systems have been identified in *L. lactis*: (i) a proton motive force-driven di-tripeptide carrier (DtpT) (Smid *et al.*, 1989a; Kunji *et al.*, 1993; Hagting *et al.*, 1994) and (ii) an ATP-driven oligopeptide transport system (Opp) that is capable of transporting peptides of 4 and up to at least 8 residues (Kunji *et al.*, 1993; Tynkkynen *et al.*, 1993).

On the basis of the size of the majority of the fragments formed from the hydrolysis of caseins by the proteinase PrtP, peptidases have been implicated in the further hydrolysis of the peptides outside the cell (Smid *et al.*, 1991; Pritchard and Coolbear, 1993). Extracellular peptidases would allow the cells to utilize caseins more efficiently and completely. The apparent discrepancy between the need for extracellular peptidases and the experimental data supporting an intracellular location of the enzymes has led us to investigate the β-casein utilization in genetically well defined peptide transport mutants expressing the P_1-type proteinase of *L. lactis* NCDO712. The analysis of the intracellular and extracellular amino acid and peptide pools has revealed that all essential and growth-stimulating amino acids can be taken up by the cells via the Opp system. Remarkably, none of the amino acids accumulated significantly inside the cell when the Opp system was inactivated. These observations provide direct evidence that peptidases are not involved in extracellular degradation and subsequent utilization of β-casein by *L. lactis*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions—The bacterial strains and plasmids used in this study are listed in Table I. Lactococcal strains were grown at 28 °C in M17 broth (Difco) or in a chemically defined medium (CDM) (Poolman and Konings, 1988) at pH 6.6, supplemented with 0.5% (w/v) glucose or lactose and when appropriate with erythromycin (5 μg/ml). The strains were stored at −20 °C in M17 broth with 10% glycerol.

General DNA Techniques—Plasmid DNA and chromosomal DNA from *L. lactis* were isolated by the methods of Anderson and McKay (1983). *L. lactis* was transformed by electroporation as described before

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TABLE I
Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics[a]	Source/references
Bacterium		
L. lactis subsp. *lactis*		
MG611	Contains multiple copies of PrtM/PrtP on the chromosome	Leenhouts *et al.* (1991)
NCDO712	Lac^+ Prt^+ $DtpT^+$ Opp^+; wild type	Gasson (1983)
MG1363	Lac^- Prt^- $DtpT^+$ Opp^+; plasmid-free derivative of NCDO712	Kunji *et al.* (1993)
MI1	Em^R, MG1363/pGK13	This work
VS772	Em^R, Lac^- Prt^- $DtpT^+$ Opp^-; MG1363 *oppA*::pLS19A	Tynkkynen *et al.* (1993)
AG300	Em^R, Lac^- Prt^- $DtpT^-$ Opp^+; MG1363 Δ*dtpT*	Hagting *et al.* (1994)
CV4	Em^R, Lac^- Prt^- $DtpT^-$ Opp^-; MG1363 Δ*dtpT oppA*::pLS19A	This work
MI2	Lac^+ Prt^+ $DtpT^+$ Opp^+; MI1/pLP712	This work
EG110	Lac^+ Prt^+ $DtpT^+$ Opp^-; VS772/pLP712	This work
EG135	Lac^+ Prt^+ $DtpT^-$ Opp^+; AG300/pLP712	This work
EG165	Lac^+ Prt^+ $DtpT^-$ Opp^-; CV4/pLP712	This work
Plasmid		
pVS8	Plasmid containing *pepO* and the *Opp* operon, Cm^R	Tynkkynen *et al.* (1993)
pGK13	Shuttle vector carrying the Em^R and Cm^R genes	Kok *et al.* (1984)
pLP712	Conjugative plasmid of NCDO712 containing the *lac* and *prtP/prtM* genes	Gasson (1983)
pLS19A	Integration vector for the disruption of *oppA*	Tynkkynen *et al.* (1993)

[a] Lac^+, lactose-fermenting phenotype; Prt^+, ability to produce a functional proteinase; $DtpT^-$, di-tripeptide transport deficient; Opp^-, oligopeptide transport deficient; Cm^R and Em^R, resistance to chloramphenicol and erythromycin, respectively.

(Holo and Nes, 1989). DNA modification enzymes were obtained from Boehringer GmbH (Mannheim, Germany). Southern hybridizations were performed using the digoxygenin DNA labeling and detection kit according to the instructions of the manufacturer (Boehringer).

Di-tripeptide and Oligopeptide Transport-deficient Mutants of L. lactis—Using *L. lactis* AG300 as parent strain (Table I), plasmid pLS19A, containing a 1,130-base pair internal fragment of *oppA* ligated into pLS19 (Leenhouts *et al.*, 1990), was used to inactivate the *oppA* gene (*oppA* encodes the oligopeptide binding protein). Disruption of the *oppA* gene was confirmed by Southern analysis. The double mutant Δ*dtpT oppA*::pLS19A was designated *L. lactis* CV4. The wild type strain (MG1363) and the various peptide transport mutants (AG300, VS772, and CV4) were conjugated with plasmid pLP712 (carrying the proteinase and lactose utilization genes) as described (Gasson and Davies, 1980).

Enzyme Assays—The proteinase activity of exponentially growing acceptor and conjugated strains was assayed with MeO-Suc-Arg-Pro-Tyr-pNA as substrate (final concentration of 0.5 mM) (Chromogenix, Mölndal, Sweden) in 80 mM Tris-HCl, pH 7.0, containing 10 mM $CaCl_2$, essentially as described (Exterkate, 1990).

Growth Experiments—To analyze growth in milk, exponentially growing cells were inoculated (at A_{660} 0.05) in 10% (w/v) skimmed milk (Oxoid, Basingstoke, United Kingdom) supplemented with 80 mM phosphoglycerate, pH 6.7. To prevent breakdown of caseins due to extensive heating, milk (and other casein-containing media) were heated to 100 °C followed by rapid cooling to 4 °C. Growth rates in milk were determined by diluting samples 10-fold in an EDTA-borate buffer and measuring the absorbance at 660 nm after 5 min (Hugenholtz and Veldkamp, 1985). Growth of *L. lactis* on peptides was tested in CDM (Poolman and Konings, 1988), containing all essential amino acids except for one, which was supplied in the form of a di- or pentapeptide. The limiting factors for growth on β-casein were identified by adding individual or combinations of essential or growth-stimulating amino acids (2 mM) to CDM containing 0.3 mM β-casein as sole source of protein. Growth was monitored by measuring changes in absorbance at 660 nm.

Transport Assays—Prior to transport, cells were washed with 100 mM potassium-MES,[2] pH 6.5, containing 2 mM $CaCl_2$ to prevent autoproteolysis and release of the proteinase (Laan and Konings, 1989). To inhibit protein synthesis, chloramphenicol (50 μg/ml) was present in all further steps. Cells (A_{660} ± 25) were de-energized with 2-deoxyglucose (10 mM) for 20 min at 30 °C, washed twice with MES/$CaCl_2$, and resuspended in 100 mM MES, pH 6.5, without $CaCl_2$. For transport assays, cells (A_{660} ± 10) were preincubated for 3 min in the presence of 25 mM glucose, after which 0.3 mM β-casein, peptides (0.5 mM), or a mixture of amino acids mimicking the concentration of 0.3 mM β-casein were added. Transport was monitored by determining the intracellular amino acid pools at various time intervals as described (Kunji *et al.*, 1993).

Cell Lysis and Viability—Samples were taken in parallel with the transport assays, cells were removed by filtration (0.45 μm of cellulose-nitrate), and the filtrate was incubated with lysyl-pNa (10 mM) for several hours at 30 °C. Lysyl-pNa is a chromogenic substrate that is specific for aminopeptidases, and its hydrolysis was measured as described (Tan and Konings, 1990). The aminopeptidase activities were related to those of sonicated cell samples (3 × 15 s at an amplitude of 6 μm, on ice and under N_2 atmosphere). Propidium iodide (Fluorescent Probes, Eugene, OR) fluorescence was used to measure the viability of cells during the experiments. The probe interacts with DNA only when the permeability barrier of the cytoplasmic membrane is disrupted. Samples were taken during the course of the experiments, propidium iodide was added (1 μg/ml), and the fluorescence before and after sonication was compared (excitation and emission wavelengths of 290 and 605 nm, respectively). Since sonication does not result in 100% lysis, it can be expected that quantification of lysis determined in this way is an overestimation.

Miscellaneous—The P_1-type proteinase of *L. lactis* was purified from strain MG611 according to Juillard *et al.*[1] Protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard. Growth experiments, peptidase, and proteinase activity determinations were performed in low protein binding ELISA plates (Greiner). Changes in absorption were measured at the appropriate wavelengths in a Titertek MC600 ELISA spectrophotometer (Flow Laboratories). To prevent evaporation during the incubations at 30 °C, the incubation mixtures (200 μl) were covered with 50 μl of silicon oil (1.01 mg/ml) (Wacker).

Chemicals—Peptides were obtained from Bachem Feinchemikalien AG (Switzerland), and β-casein was from Sigma. All amino acids were in the L configuration. Milli-Q water (Millipore Corp.) was used in all experiments.

RESULTS

Characterization of the Peptide Transport Mutants—The phenotypes of the wild type and peptide transport mutants (MI2, EG110, EG135, EG165) (Table I) were checked by different criteria. First, growth of the organisms on specific peptides was measured using a chemically defined medium containing all essential amino acids except for one, which was supplied in the form of Ala-Glu or Tyr-Gly-Gly-Phe-Leu (Table II) (amino acids omitted from the medium are underlined). The strains lacking a functional DtpT system failed to grow in the presence of Ala-Glu as sole source of glutamate, while strains in which the Opp system was inactivated did not grow on Tyr-Gly-Gly-Phe-Leu as sole source of leucine. All strains grew equally well in the presence of amino acids as essential and growth-stimulating nutrients. Second, the uptake of substrates that are specific for the Ala/Gly, the di-tripeptide, and oligopeptide transport systems was studied (Table II). In accordance with

[2] The abbreviations used are: MES, 2-(*N*-morpholino)ethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography.

TABLE II
Characterization of L. lactis wild type and peptide transport mutants

	MI2 (Prt$^+$ DtpT$^+$ Opp$^+$)[a]	EG110 (Prt$^+$ DtpT$^+$ Opp$^-$)	EG135 (Prt$^+$ DtpT$^-$ Opp$^+$)	EG165 (Prt$^+$ DtpT$^-$ Opp$^-$)
Maximal growth rate (h^{-1})[b]				
Amino acids	0.92	0.94	0.99	0.96
Ala-Glu	0.76	0.72	0.00	0.02
Tyr-Gly-Gly-Phe-Leu	0.76	0.00	0.69	0.03
Alanine uptake rates[c]				
Alanine	55	56	65	75
Di-alanine	108	120	1	1
Tetra-alanine	21	2	25	1
Alanyl-β-chloro-alanine[d]	Sensitive	Sensitive	Resistant	Resistant

[a] Strain numbers are followed by phenotypes in parentheses.
[b] Maximal growth rates (h^{-1}) have been calculated from the increase in A_{660} in chemically defined medium containing all amino acids or all amino acids minus Glu plus Ala-Glu or all amino acids minus Leu plus Tyr-Gly-Gly-Phe-Leu, as indicated.
[c] Initial rates of accumulation of alanine (nmol/min·mg of protein) with Ala, Ala-Ala (di-alanine), or Ala-Ala-Ala-Ala (tetra-alanine) as substrates (0.5 mM each).
[d] Sensitivity to the toxic dipeptide alanyl-β-chloro-alanine (250 μM) when present in a chemically defined medium.

the growth experiments, the di-alanine was taken up when DtpT was functional, and tetra-alanine was transported when the Opp system was functional, whereas high uptake rates of alanine were observed in all four strains. Third, the presence or absence of a functional di-tripeptide transport system was inferred from the sensitivity of the strains for the toxic dipeptide Ala-β-chloro-Ala (250 μM) (Table II). Strains containing the di-tripeptide transport system were sensitive to the toxic dipeptide, while strains in which the system was absent were resistant. Fourth, the proteinase activity of the acceptor and conjugated strains was assayed by monitoring the hydrolysis of the chromogenic substrate MeO-Suc-Arg-Pro-Tyr-pNA (Fig. 1*A*). High proteinase activities were present in all conjugated strains, while no activity was observed in the acceptor strains. The specific proteinase activities corresponded to 1.9, 2.1, 3.8, and 4.8 nmol/min·mg of protein for the Opp$^+$ DtpT$^+$, Opp$^-$ DtpT$^+$, Opp$^+$ DtpT$^-$, and Opp$^-$ DtpT$^-$ strains, respectively. Fifth, the growth properties of the conjugated strains in milk were studied by monitoring the change in optical density and pH (Fig. 1*B*). The results clearly indicate that oligopeptide transport is essential for growth on milk. Strains lacking a functional di-tripeptide transport system, on the other hand, grew equally well as the wild type. Slow growth of the oligopeptide transport mutants in milk was observed after 24 h, possibly due to cell lysis followed by release of peptidases into the medium.

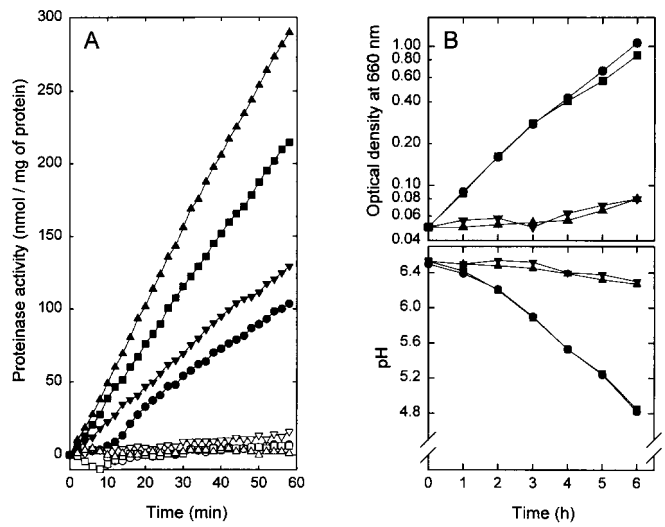

FIG. 1. **The proteinase activity (*A*) and growth on milk (*B*) of *L. lactis* wild type and peptide transport mutants.** The proteinase activity of *L. lactis* MI1 (○), VS772 (▽), AG300 (□), and CV4 (△) and the corresponding conjugants carrying pLP712 (*closed symbols*) was assayed with the chromogenic substrate MeO-Suc-Arg-Pro-Tyr-pNA by measuring the change in absorption at 414 nm upon hydrolysis of the substrate. Growth on skimmed milk was determined as indicated under "Materials and Methods."

β-Casein Utilization by the Wild Type and Peptide Transport Mutants—The effect of the various peptide transport mutations on the utilization of β-casein was studied *in vivo* by incubating chloramphenicol-treated cells with 0.3 mM β-casein in the presence of glucose as source of metabolic energy. At given time intervals, cells were separated from the medium by filtration, and the cell fraction was extracted with perchloric acid. Both the extra- and intracellular fractions were analyzed by reverse-phase HPLC for the presence of amino acids and peptides after derivatization with dansylchloride.

Fig. 2 shows the time course of the intracellular amino acid pools (1–7) for the Opp$^+$ DtpT$^+$ (*A*), Opp$^-$ DtpT$^+$ (*B*), Opp$^+$ DtpT$^-$ (*C*), and Opp$^-$ DtpT$^-$ (*D*) strains upon the addition of β-casein. The wild type and di-tripeptide transport mutant rapidly accumulated almost all of the amino acids present in β-casein within minutes after the addition of the protein substrate (Fig. 2, *A* and *C*). The rates of amino acid accumulation were in case of the di-tripeptide transport mutant on average 1.6 times higher as compared with the wild type, which might reflect the higher proteinase activity (Fig. 1*A*) and/or the higher oligopeptide transport activity of the strain (Table II). Remarkably, none of the amino acids from β-casein accumulated to significant levels in the strains lacking a functional oligopeptide transport system, despite the presence of functional amino acid and/or di-tripeptide transport systems (Fig. 2, *B* and *D*). The observation that a single mutation, abolishing oligopeptide transport activity, resulted in a defect to accumulate amino acids argues strongly against degradation of peptides by extracellularly located peptidases. In the analysis of the elution profiles from the intracellular fraction, almost all of the peaks could be attributed to amino acids, whereas significant amounts of peptides could not be detected (see also Kunji *et al.*, 1993; Tynkkynen *et al.*, 1993; Hagting *et al.*, 1994).

A number of observations regarding the accumulation of amino acids upon hydrolysis of β-casein deserve further attention. Due to the high intracellular pools of Glu, Asp could not be separated well from Glu, and the sum of both pools is represented by Glu in Fig. 2, *A1–D1*. Furthermore *L. lactis* converts Gln into Glu and Asn into Asp (Poolman *et al.*, 1987a), and therefore, the "Glu" peak forms an indication of the accumulation of Glu, Gln, Asp, as well as Asn. His might also be converted since the pools initially drop upon energization in all four strains (Fig. 2, *A6–D6*). With the exception of Gln to Glu,

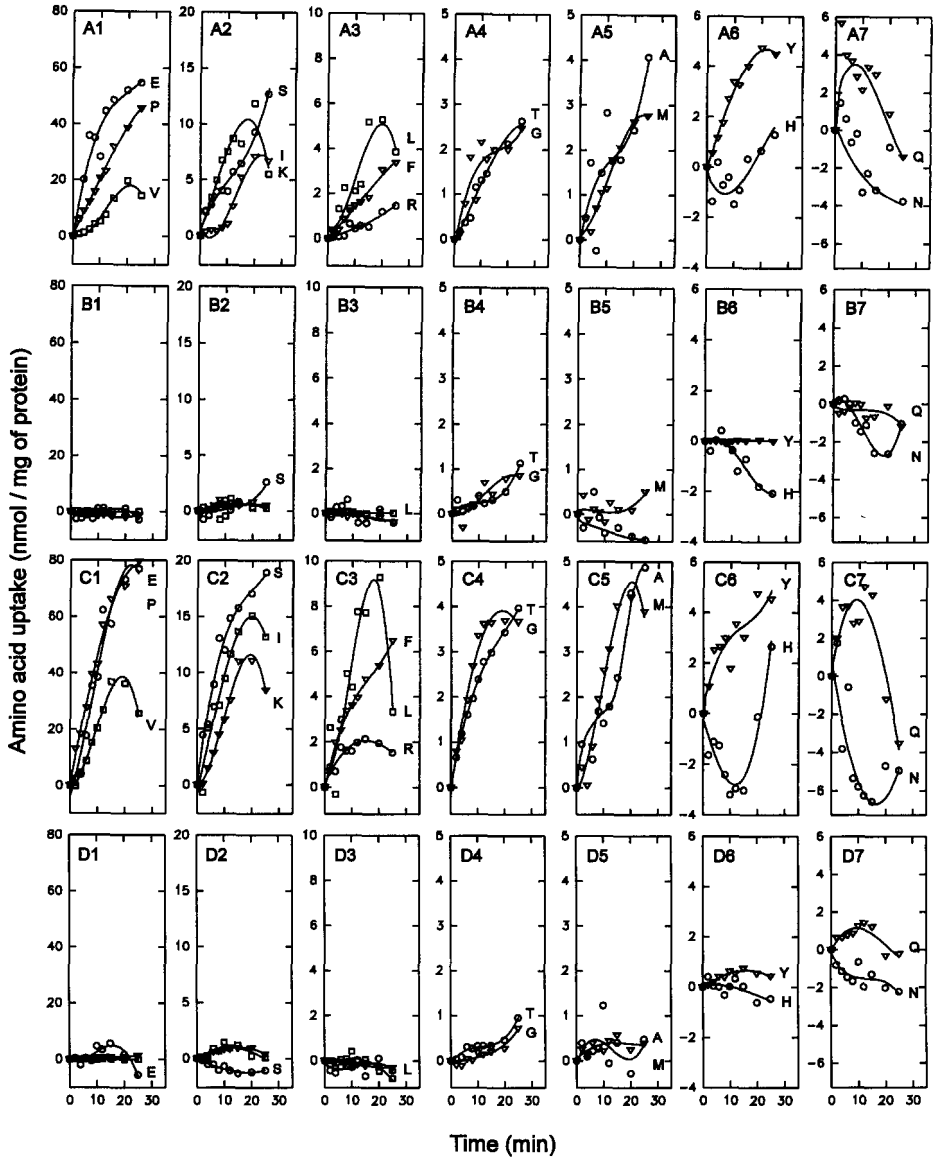

FIG. 2. **Time course of intracellular amino acid accumulation (*1–7*) for the wild type (*A*), the oligopeptide transport mutant (*B*), the di-tripeptide transport mutant (*C*), and the double mutant (*D*) upon the addition of β-casein.** De-energized and chloramphenicol-treated cells were incubated with 0.3 mM β-casein after 3 min of pre-energization with 25 mM glucose as source of metabolic energy. The amino acid pools were determined as described under "Materials and Methods" and are indicated by their one letter denomination. The increase in amino acid concentration compared with the concentration at $t = 0$ min is depicted. The time 0 concentrations for the amino acids were less than 5 nmol/mg for Asn, Gln, Ser, Arg, Thr, Gly, Pro, Met, Val, Phe, Leu, Ile, His, Lys, and Tyr and 19 and 52 nmol/mg for Ala and Glu, respectively.

Asn to Asp, and Arg to ornithine and citrulline (Poolman *et al.*, 1987b), *L. lactis* has no possibilities to convert amino acids into other compounds under the conditions employed. Furthermore, using amino acid-depleted resting *L. lactis* cells, we have never detected significant synthesis of amino acids from precursors of the glycolytic pathway (Poolman *et al.*, 1987a; this study). Therefore, we conclude that, with the possible exception of Gly and Thr (see Fig. 2, *B4* and *D4*), the increases in amino acid pools in the Opp$^+$ strains (Fig. 2, *A* and *C*) result from the uptake of the corresponding amino acids in the form of oligopeptides.

Quantification of Cell Lysis—The amount of cell lysis occurring during the course of the transport assays was estimated from the aminopeptidase activities and viability of the culture. Aminopeptidases PepC and PepN have been characterized as highly active intracellular enzymes (Tan *et al.*, 1992), and their activities could therefore be used as markers for cell lysis. No detectable lysis was observed at the start of the experiment, probably due to extensive washing prior to the transport assays. After 15 min of incubation, some peptidase activity could be detected corresponding to at most 1% cell lysis; significant differences between the wild type and peptide transport mutants could not be detected. The viability of the cell suspensions remained constant during the course of the experiments (data not shown). All together, these experiments show that the observed differences in β-casein utilization between strains with and without a functional Opp system are not a consequence of differences in cell lysis (and subsequent release of intracellular peptidases).

Growth of the Wild Type and Peptide Transport Mutants on β-Casein—It is worthwhile noting that at least six amino acids (Glu/Gln, Leu, Val, Ile, Met, His) are essential for *L. lactis* MG1363, whereas another four (Asn, Pro, Phe, and Ala) are needed to support reasonable rates of growth.[3] The wild type and DtpT$^-$ mutant of *L. lactis* grew slowly and only to low final optical densities on 0.3 mM β-casein as sole source of amino acids, indicating that one or more essential amino acids are not liberated or transported sufficiently fast to support optimal growth (data not shown). By adding individual or combinations of essential and growth-stimulating amino acids to media containing β-casein as protein source of amino acids, the limiting factors for optimal growth on β-casein alone could be identified. These experiments showed that the addition of His and Leu (2

[3] E. R. S. Kunji, unpublished results.

mM each) was sufficient for growth of the wild type and di-tripeptide transport mutant on 0.3 mM β-casein; the maximal growth rates (μ_{max}) were 0.63 h^{-1} for both strains. Subsequent additions of Gln, Val, and Met (2 mM) were found to be growth stimulatory. The μ_{max} values increased to 0.83, 0.86, and 0.89 h^{-1}, respectively. On the contrary, the oligopeptide transport mutants did not grow under any of the conditions (*i.e.* media containing β-casein plus indicated amino acids). Only when a complete mixture of amino acids was added, cells grew with a high rate (μ_{max} of 0.93 h^{-1}). This again confirms that the total package of amino acids derived from β-casein is exclusively taken up as oligopeptides.

Amino Acid Transport in the Wild Type and Peptide Transport Mutant Strains—The various strains were fed with a mixture of amino acids mimicking the composition of β-casein (Tyr was omitted). The pool sizes of individual amino acids increased to similar extents, and the rates of uptake were not significantly different in all four strains (data not shown). These results demonstrate that the inability of the oligopeptide transport mutants to accumulate β-casein-derived amino acids is not due to some general defect of the organisms to transport solutes.

The External Accumulation of Peptides and Amino Acids—To obtain further information about the role of the Opp system in β-casein utilization, the hydrolysis products of β-casein generated by the purified proteinase PrtP and by cells expressing PrtP have been compared. The peptide product formation from the hydrolysis of β-casein *in vivo* has been studied by analyzing the external media of the uptake experiments described in Fig. 2. The β-casein degradation patterns obtained with the purified proteinase were similar to those of the *in vivo* experiments, in which the Opp^- strains EG110 and EG165 were used (data not shown). Pronounced differences in peptide pools can be observed when strains with a functional oligopeptide transport system are compared with those lacking a functional oligopeptide transport system. At least 18 peaks were observed in the media of Opp mutants, which were virtually absent in the media of strains with an active Opp (data not shown). These peptides are likely to be substrates of the oligopeptide transport system. Furthermore, only when the oligopeptide transport system was functional, several peaks increased rapidly, of which most could be assigned to individual amino acids. Notice that peptides, taken up by *L. lactis*, are rapidly hydrolyzed by intracellular peptidases, which results in large outwardly directed concentration gradients for the various amino acids. As a result, amino acids will leak from the cells and appear in the medium. The estimates are that less than 5% of the internally accumulated amino acids had effluxed from the cells at the end of the experiment.

DISCUSSION

The experiments described in this study have revealed a number of important properties of the proteolytic pathway of *L. lactis*. First, all of the essential and growth-stimulating amino acids for *L. lactis* can be released from β-casein by the action of the proteinase PrtP in a form that can be transported by the cells. Second, these peptides are taken up by the oligopeptide transport system exclusively. When a functional oligopeptide transport system is absent, no significant intracellular accumulation of amino acids is observed. Under those circumstances, several peptides accumulate extracellularly, which do not accumulate when the oligopeptide transport system is functionally present. Third, consistent with the observation that PrtP does not release significant amounts of di- and tripeptides from β-casein,[1] inactivation of the di-tripeptide transport system has no effect on the utilization of this protein substrate. Since di-tripeptide transport mutants selected on the basis of resistance toward L-Ala-β-chloro-L-Ala are affected in their ability to grow on a mixture of caseins (Smid *et al.*, 1989b), we speculate that this is due to the inability to transport essential amino acids (most likely His and/or Leu, see below) in the form of small peptides that are released from proteins other than β-casein. Fourth, the observation that a single mutation abolishing oligopeptide transport activity results in a defect to accumulate amino acids argues strongly against the involvement of extracellular peptidases in the degradation of β-casein. If peptidases would have been present externally, amino acids, dipeptides, and tripeptides would have been formed and subsequently taken up by the corresponding transport systems. Biochemical and genetic studies on a number of peptidases have already suggested that these enzymes are present intracellularly (Tan *et al.*, 1992; Kok and De Vos, 1993). The present studies indicate that also other not yet identified or poorly characterized peptidases, involved in β-casein utilization, are unlikely to be present extracellularly. Since synthetic peptides such as Leu-enkephaline, tetra-alanine, Ala-Glu, and others are not degraded extracellularly irrespective of whether Opp^+ or Opp^- strains are used (Kunji *et al.*, 1993),[3] it is unlikely that Opp-mediated regulation of the function or expression of extracellular proteases/peptidases has effected the experiments. Fifth, a small fraction of the intracellularly accumulated amino acids appears in the extracellular medium of the wild type and di-tripeptide transport mutant, most likely due to leakage of amino acids from the cells. Sixth, the observation that the wild type and di-tripeptide transport mutant of *L. lactis* grow well on a chemically defined medium supplemented with β-casein and histidine plus leucine as sole source of amino acids indicates that uptake of the oligopeptides occurs at rates high enough to meet the growth requirements of the organism for most of the (essential) amino acids. This is quite remarkable given the competition between the peptides for a single binding protein. Rapid accumulation of most amino acids is observed within minutes after the addition of β-casein, indicating that proteolysis and oligopeptide transport are indeed quite effective.

A large number of peptides are released from β-casein by the activity of the proteinase.[1] Both from the analysis of the extracellular as well as the intracellular fractions, it can be concluded that not all proteinase-generated peptides are utilized by *L. lactis*. On the basis of the differences in peptide patterns in the external medium of the Opp^+ and Opp^- strains, one-fourth of all peptide peaks observed in the HPLC analysis of the peptide pools are likely to contain substrates of the oligopeptide transport system. The observation that some peptides do accumulate in the medium despite a functional Opp system may be a consequence of the size exclusion limits of the oligopeptide transporter. Peptides up to a length of 30 amino acids are formed by PrtP.[1] Approximately one-fifth of the β-casein-derived peptides falls in the range of 4–8 residues,[1] and these are likely to be transported (Kunji *et al.*, 1993; Tynkkynen *et al.*, 1993). Furthermore, although the lactococcal oligopeptide transport system must have a broad substrate specificity, certain peptides may not be transported due to competition of peptides for a single oligopeptide binding protein. In addition, a part of the peptide pool may also be taken up with a rate that is lower than the production rate by the proteinase.

In future studies, we aim to identify the time course of peptide product formation and the size restriction and substrate specificity of the oligopeptide transport system for its natural substrates. It is always assumed that peptide transport systems do not transport fragments longer than 5 or 6 residues (Payne and Smith, 1994). An intriguing aspect is the observa-

tion that the Opp system of *L. lactis* transports peptides with a size of at least 8 residues (Tynkkynen *et al.*, 1993), but perhaps the transportable species can even be longer.

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REFERENCES

Anderson, D. G., and McKay, L. L. (1983) *Appl. Environ. Microbiol.* **46,** 549–552
Baankreis, R. (1992) *The Role of Lactococcal Peptidases in Disease Ripening*, Ph.D. thesis, University of Wageningen
Exterkate, F. (1990) *Appl. Microbiol. Biotechnol.* **33,** 401–406
Gasson, M. J. (1983) *J. Bacteriol.* **154,** 1–9
Gasson, M. J., and Davies, F. L. (1980) *FEMS Microbiol. Lett.* **7,** 51–53
Hagting, A., Kunji, E. R. S., Leenhouts, K. J., Poolman, B., and Konings, W. N. (1994) *J. Biol. Chem.* **269,** 11391–11399
Holo, H., and Nes, I. F. (1989) *Appl. Environ. Microbiol.* **55,** 3119–3123
Hugenholtz, J., and Veldkamp, H. (1985) *FEMS Microbiol. Ecol.* **31,** 57–62
Kok, J., and De Vos, W. M. (1993) in *Genetics and Biotechnology of Lactic Acid Bacteria* (Gasson, M. J., and De Vos, W. M., eds), pp. 169–210, Blackie Academic & Professional, London
Kok, J., Van der Vossen, J. M. B. M., and Venema, G. (1984) *Appl. Environ. Microbiol.* **48,** 726–731
Kunji, E. R. S., Smid, E. J., Plapp, R., Poolman, B., and Konings, W. N. (1993) *J. Bacteriol.* **175,** 2052–2059
Laan, H., and Konings, W. N. (1989) *Appl. Environ. Microbiol.* **55,** 3101–3106
Leenhouts, K. J., Kok, J., and Venema, G. (1990) *Appl. Environ. Microbiol.* **56,** 2726–2735
Leenhouts, K. J., Gietema, J., Kok, J., and Venema, G. (1991) *Appl. Environ. Microbiol.* **57,** 2568–2575
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
Payne, J. W., and Smith, M. W. (1994) *Advances Microb. Physiol.* **36,** 1–80
Poolman, B., and Konings, W. N. (1988) *J. Bacteriol.* **170,** 700–707
Poolman, B., Smid, E. J., and Konings, W. N. (1987a) *J. Bacteriol.* **169,** 2755–2761
Poolman, B., Driessen, A. J. M., and Konings, W. N. (1987b) *J. Bacteriol.* **169,** 5597–5604
Pritchard, G. G., and Coolbear, T. (1993) *FEMS Microbiol. Rev.* **12,** 179–206
Smid, E. J., Driessen, A. J. M., and Konings, W. N. (1989a) *J. Bacteriol.* **171,** 292–298
Smid, E. J., Plapp, R., and Konings, W. N. (1989b) *J. Bacteriol.* **171,** 6135–6140
Smid, E. J., Poolman, B., and Konings, W. N. (1991) *Appl. Environ. Microbiol.* **57,** 2447–2452
Tan, P. S. T., and Konings, W. N. (1990) *Appl. Environ. Microbiol.* **56,** 526–532
Tan, P. S. T., Capot-Chartier, M.-P., Pos, K. M., Rousseau, M., Boquien, C. Y., Gripon, C., and Konings, W. N. (1992) *Appl. Environ. Microbiol.* **58,** 285–290
Tynkkynen, S., Buist, G., Kunji, E., Kok, J., Poolman, B., Venema, G., Haandrikman, A. (1993) *J. Bacteriol.* **175,** 7523–7532